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International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C12N 15/62, 9/38, 15/75
C07K 7/10

A1

(11) International Publication Number: WO 93/03156

(43) International Publication Date: 18 February 1993 (18.02.93)

(21) International Application Number: PCT/US92/05810

(22) International Filing Date: 9 July 1992 (09.07.92)

(30) Priority data:
736,447 26 July 1991 (26.07.91) US

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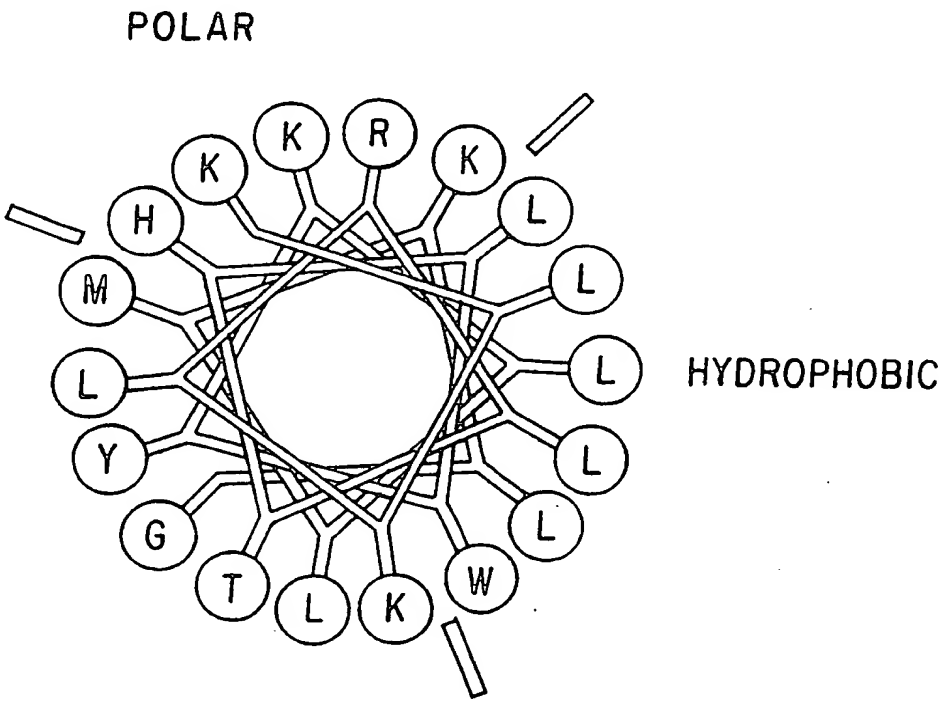
(81) Designated States: AT, AU, BB, BG, BR, CA, CH, CS, DE, DK, ES, FI, GB, HU, JP, KP, KR, LK, LU, MG, MN, MW, NL, NO, PL, RO, RU, SD, SE, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LU, MC, NL, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, SN, TD, TG).

Published
With international search report.

(54) Title: SEQUENCE FOR STABILIZING PROTEINS IN BACTERIA PROTEIN STABILIZATION SEQUENCE

(57) Abstract

The invention relates to a protein stabilizing sequence particularly useful for stabilization of proteolytically sensitive proteins. The sequence includes a relatively small number of amino acids that may be expressed fused with a proteolytically sensitive protein. The most effective stabilization sequences assume α-helix structures with a hydrophobic face and a positively charged polar face which appear to require proper orientation with respect to each other. Other aspects of the invention include cloning vectors incorporating a gene sequence encoding the stabilization polypeptide and production of stabilized antigenic proteins.



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DESCRIPTION

SEQUENCE FOR STABILIZING PROTEINS IN BACTERIA

PROTEIN STABILIZATION SEQUENCE

The United States Government may have certain rights in the present invention pursuant to the terms of Grant No. 86-13978 awarded by the National Science Foundation.

BACKGROUND OF THE INVENTION

Field of the Invention

The invention relates to an amino acid sequence useful for stabilization of otherwise unstable proteins, particularly proteolytically sensitive proteins. The invention also relates to methods of producing stabilized proteins by direct attachment of the stabilizing sequence or through fusion proteins expressed from recombinant host cells.

Description of Related Art

One of the major problems in the use and preparation of therapeutic and commercial proteins is degradation by cell proteases. This may occur *in vivo* in plasma or inside a host cell when recombinant methods of polypeptide production are employed. Degradation plays an obvious role in the elimination of damaged or abnormal proteins but also affects half lives of normal proteins. Individual protein turnover rates may vary 1000 fold, depending on environmental and structural factors.

Two general factors contribute to the half-life of any given protein. One factor includes "global" features such as large size, hydrophobicity, thermal instability and charge characteristics. The other factor is related to sequence specific parameters of particular α-amino

terminii, asparagine residues and so-called PEST sequences. Although correlations have been made between particular features and susceptibility to degradation, explicit degradation signals and pathways are not well defined. It is not even known whether peptide bond cleavage or biochemical marking is the primary event signaling protein destruction.

In contrast to degradation factors, there appear to be stabilization factors that enhance stability of a protein toward proteolysis. It has been suggested that both thermodynamic stability and proteolytic susceptibility are major determining factors in rate of degradation (Parsell and Sauer, 1989). There has been some evidence that particular sequences at the N-terminus of lambda repressor in *Escherichia coli* aid in maintaining a stable tertiary structure and therefore slow proteolytic degradation (Parsell and Sauer, 1989). Some evidence also suggests that the increased stability of certain fusion proteins may derive from the ability of a stable fusion partner such as protein G or β-galactosidase to stabilize the tertiary structure of a proteolytically sensitive protein (Héllebust *et al.*, 1989).

A particular amino acid sequence attached as a "tail" to the C-terminal of Arc, a small dimeric DNA-binding protein found in bacteriophage P22, has been found to stabilize that protein against proteolysis. The primary sequence of the tail consists of 25 amino acids which when attached to a heterologous protein, the LP57 mutant of λ-repressor in *Escherichia coli*, also stabilized that protein (Bowie and Sauer, 1989).

Nevertheless, up until now, an amino acid sequence attachable to a proteolytically sensitive protein has not been found which will generally increase stability of

these proteins. The value of such a sequence would lie in its ability to increase the half-life of commercially desired proteins for production in recombinant cell hosts and the potential to protect proteinaceous vaccines from serum protease degradation when injected directly into an animal.

SUMMARY OF THE INVENTION

The invention generally relates to a protein stabilization sequence. The sequence is comprised of amino acids that may be attached to proteolytically sensitive proteins.

The stabilization sequence includes a limited number of amino acids ranging from about ten to about 50 residues. The amino acids need not be confined to a particular type of amino acid; however, the amino acid residues will be such that the secondary and tertiary structure assumes the form of an outwardly directed, properly aligned hydrophobic face and a positively charged polar face. The importance of this structure has been shown by substituting two of the amino acids causing the structure to have a twisted form. This form was shown to be less stable. The alignment of amino acid groups within a particular α-helical form appears to be important in order for the amino acid sequence to have maximal effect as a stabilization sequence.

The α-helical structure assumed by the amino acid protecting sequence is an important aspect of the invention. Thus it is number and type of amino acid residues involved, and the structure assumed by the amino acid residues making up the structure. Twisted forms of the α-helix structure with the hydrophobic and polar faces out of alignment provide less stability to fused

heterologous proteins. Thus in preferred embodiments the outwardly directed hydrophobic face of the structure is in alignment with positively charged face of the α-helix.

Although it is believed that such stabilization structures could be formed with as many as a 100 amino acid residues, the amino acid sequence used is preferably from about 10 to about 50 acid residues and more preferably 29 amino acid residues. A particularly preferred amino acid sequence is shown in Figure 1.

Another aspect of the invention is the attachment of the protein stabilization sequence to a proteolytically sensitive enzyme. Such attachments may be made at the time of synthesis of the proteolytically sensitive protein of interest or alternatively may be used in *in vitro* situations where it is desired to protect a proteolytically sensitive protein from degradation. An example might include the use of the protecting sequence to protect an antigenic protein that is being used to promote antibody formation *in vivo*. Protection of the antigen from serum proteases would provide more effective use of expensive antigenic materials that are in short supply.

In a preferred embodiment, the protein stabilization sequence is prepared in the form of a cassette in which the DNA segment encoding the sequence is incorporated into a cloning vector. Proper construction of the vector results in a protein of interest being expressed with the polypeptide protecting group attached to the protein, thus protecting it from protease degradation within the host cell in which it is expressed.

Attachment of the protein stabilization sequence is generally to the amino-terminal end of the proteolytically sensitive protein or to the carboxy

terminal end. In some cases, for example, exceptionally long proteolytically sensitive proteins it may be desirable to attach the stabilization sequence to both termini. *In vitro* attachment methods may be readily accomplished using chemical attachments well known to those of skill in the art. Attachment of such sequences *in vivo* is possible by constructing appropriately designed cloning rectors.

The protein stabilization sequence described has general utility and is not limited to stabilization of the polymerase sigma factor from which it was originally derived. RNA polymerase sigma factors from *B. subtilis* or *E. coli* have been prepared as fusion proteins with the stabilization factor originally isolated from *B. subtilis* sigma factor. It is envisioned that virtually any DNA segment encoding a polypeptide could be constructed in a cloning vector or expression vector with the DNA segment encoding the stabilization factor sequence. Examples of other proteolytically sensitive proteins that might be protected with the stabilization sequence include proinsulin, epidermal growth factor, interleukin, interferon, somato tropin, insulin-like growth factor, phosphatase, immunoglobulin Fv and the like. Moreover expression may be from a variety of suitable bacterial cells provided that they can be suitably transformed by an appropriately constructed cloning vector. Examples of suitable host microorganisms include *E. coli, B. subtilis, S. tymphyrium* and the like.

The invention is also envisioned as a method of enhancing stability of recombinant proteins. A first step would be to obtain a gene segment encoding the protein stabilization sequence. Such a sequence is not limited to a particular number or type of amino acid residues provided that the residues form the appropriate structure having the stabilization properties. Thus

under certain conditions it may be desirable to encode up to 100 amino acids in the sequence. In a further step a recombinant vector is prepared. This vector will have a gene encoding the protein stabilization sequence selected as well as a gene encoding a protein desired to be stabilized. Generally, this is accomplished by preparing a cloning site within a plasmid vector that encodes the protein stabilization sequence, then inserting into that site a gene or a gene segment encoding the protein desired to be stabilized. The cloning site may be modified to include one or more unique restriction sites but in general will be constructed so that insertion of the gene of choice will restore a proper reading frame between the protein stabilization sequence and the cloning site through the desired gene segment. Such a recombinant vector may then be used to transform a host cell. Transformed host cell colonies will then be selected, for example by incorporating selected genes within the recombinant vectors such as antibiotic resistance genes or alkaline phosphatase expressing genes which will elicit positive or negative responses depending on whether the desired gene has been cloned into the cloning site. Once transformed host cell colonies are identified, expression of a heterologous protein fused with the aforedescribed protein stabilization sequence may be affected. This is generally performed using standard culture methods well known to those of skill in the art.

Expression of a wide variety of heterologous proteins fused with the protein stabilization sequences should be possible. In general proteolytically sensitive proteins suitable for production as stable heterologous proteins from prokaryotic hosts will be proteins that are expressible as active fusion proteins. Such proteins include ß-galactosidase, sigma factor and the like.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows an amino acid sequence capable of forming a protecting hydrophobic faced α-helix structure when attached to a proteolytically sensitive protein.

Figure 2 shows the nucleic acid sequence encoding a 29 amino acid protective sequence.

Figure 3 illustrates generally the α-helix formed by amino acid sequences that confer proteolytic stability on proteins to which they are N-terminally attached. A hydrophobic face and a positively charged polar face are aligned on the a-helix.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Materials and Methods

Bacterial Strains and Plasmids

B. subtilis strains JH-642 (*pheA trpC2*) and 1A287 (*rpsl*) were obtained from J. Hoch and the *Bacillus* Genetic Stock Center (Ohio State University), respectively. SR104 has an internal deletion in *sig*E. It was created by transformation of strain SMY with DNA from CM01 (*trpC2 pheAl ΔsigE::erm*). The M13 host strains CJ236 (*dut ung*) and JM103 (*dut+ ung+*) were obtained from V. Deretic (University of Texas Health Science Center at San Antonio). *Escherichia coli* strain TB-1 was provided by D. Kolodrubetz (University of Texas Health Science Center at San Antonio). Plasmids pSR-5 (*spoIID::lacZ*) (Rong *et al.* 1986), pSI-1 (*pSpac*) (Yansura and Henner, 1984), pGSIIG11 (*sig*E) (Stragier *et al.*, 1984), and pSGMU31 (Errington, 1986) have been described, pJM102, obtained from J. Hoch, is pUC18 (Yanisch-Perron *et al.*,

1985) with a chloramphenicol acetyltransferase (*cat*: 950 base pairs [bp]) gene cloned into its unique *NdeI* site, pSGMU31Δ2 was derived from pSGMU31 by cutting the plasmid at its two *BamHI* sites and single *BgIII* site and relegating the two major fragments. This results in loss of a small, nonessential *BamHI-BgIII* fragment (150bp) and the creation of a single *BamHI* site in the vector. pSGMU31Δ2 has the *BamHI* site and the *lacZ* gene of the original plasmid downstream of the plasmid's lac promoter. pSR22 is pBR322 (Bolivar *et al.*, 1977) and pUB110 (Gryczan *et al.*, 1978) joined at their *BamHI* sites plus a 1.1-kbp fragment encoding *sigE* (Stragier *et al.* 1984). The *sigE* fragment was cloned into the *EcoRI* site of pBR322 by using *EcoRI* linkers. pSR51 and pSR54 are variants of pSR22 with missense mutations in the coding sequence of *sigE*.

Oligonucleotide-Directed Mutagenesis

The 1.1-kpb *PstI* fragment of pGSIIG11 was cloned into M13mp19 (Yanisch-Perron *et al.* 1985), and its orientation within the vector was determined by restriction endonuclease analysis of RF DNA. To construct *sigE*Δ84, an oligonucleotide (60-mer) that carried the sequence 30 bases upstream of and including the ATG codon (nucleotides 179 to 181 of the 1.1-kpb fragment) of P^{31} and 30 bases downstream of the σ^E amino terminus (nucleotides 266 to 268) was synthesized. The 60-mer was hybridized to single-stranded M13 DNA containing its complementary sequence within the cloned 1.1-kpb *PstI* fragment. The P^{31}-specific sequence was "looped out" as a consequence of using DNA polymerase I to synthesize a complement to the circular DNA by using the oligonucleotide as primer. Enrichment for the deleted sequence was accomplished by S1 nuclease treatment of the 84-base loop following the

polymerization reaction. The hybridization, polymerization, and S1 treatments were accomplished as described by Adelman et al. (Adelman *et al.* 1983). Following transformation of *E. coli* JM103, mutagenized clones were screened by restriction endonuclease analysis for reduction in the size of the cloned *PstI* fragment (i.e., 1.1 kbp to 1.02 kbp) and the loss of the *HindII* site originally present in the loop region. Approximately 5% of the resulting clones had the desired mutation.

Additional mutant clones *sig*EΔ81, *sig*EΔ48, *sig*EΔ252, and *sig*EΔ253 were constructed by using oligonucleotides of 30 bases (30-mers) (Kunkel, 1984). M13mp18 containing either the original 1.1-kbp *sig*E fragment for *sig*EΔ81 and *sig*EΔ84) or 1.02-kbp *sig*EΔ84 fragment (for *sig*EΔ252 and *sig*EΔ253) was plaque purified twice on *E. coli* CJ236 (*dut ung*) and grown on this host to incorporate uracil into the phage DNA. Following hybridization to a 30-mer, Sequenase:2.0 (United States Biochemical Corp., Cleveland, Ohio) was used to synthesize the complementary strand. Selection for the strand polymerized *in vitro* was accomplished by transforming and plating the polymerization mixture on *E. coli* JM103 (*dut+ ung+*), which selects against the uracil-containing template. From 50 to 90% of the clones arising from the reaction contained the desired mutations. These were identified by size changes in the cloned *B. subtilis* fragment and by DNA sequencing.

Strain Construction

The *E. coli lac gene* was translationally coupled to *sig*E by cloning a 340-bp *PstI-SauIIIAl* fragment of the 1.1-kbp clones *sig*E gene into *PstI-BamHI*-cut pSGMU31Δ2. This coupled the 5′ end of *lacZ* in frame to *sig*E at a site 165 bp downstream of the sequence encoding the amino

terminus of P^{31}. In a similar fashion, the 256-bp *PstI-SauIIIAI* fragment from *sigE*Δ84 was cloned into this vector. This construct was formed at the same site within *sigE* but had only 81 bp of the *sigE* protein-coding region upstream of *lacZ*. JH642 was transformed with pSGMU31Δ2 containing the coding sequence for either the *sigE*+- or *sigE*Δ84-encoded amino terminus. Clones in which the plasmid had integrated into the *B. subtilis* chromosome were selected by chloramphenicol resistance (2.5 μg/ml) and screened by Southern blot analysis for integration into the *sigE* locus with the proper *sigE* allele positioned upstream of *lacZ*.

Strains of JH642 in which the wild-type *sigE* locus had been replaced by mutant *sigE* alleles were constructed by congression of the mutant alleles, cloned as a 1- to 1.1-kbp *PstI* fragment in JM102, with a streptomycin resistance (Str^r) marker from *B. subtilis* 1A287. Str^r colonies were screened for Spo^- by failure of the colonies to run brown on DS (Schaeffer *et al.*, 1965) plates after 24 h at 37°C. Spo^- clones were then screened for Cm^s to identify those in which the mutant allele had entered the chromosome by gene replacement rather than integration of the entire plasmid. Chromosomal DNA from clones that met these criteria were screened by Southern blot analysis for the predicted gene replacement.

Analysis of Extracts for P^{31} and σ^E-like Proteins

B. subtilis cultures were grown in DS medium, cells were harvested, and protein extracts were prepared as described previously (Trempy *et al.*, 1985), except that the ammonium sulfate step was omitted. Protein samples (100 μg) (determined by the Coomassie method, Bio-Rad Laboratories) were precipitated with 2 volumes of cold ethanol, suspended in sample buffer, and fractionated on sodium dodecyl sulfate-polyacrylamide gels (12%

acrylamide). Following electrophoretic transfer to nitrocellulose and blocking of the nitrocellulose with Blotto, the protein bands were probed with an anti-P^{31} /σ^e monoclonal antibody (Trempy *et al.*, 1985). Bound antibody was visualized by using a horseradish peroxidase-conjugated goat immunoglobulin against mouse immunoglobulin (Hyclone Laboratories, Inc.) or ^{125}I-rabbit anti-mouse immunoglobulin antibody (K. Krolick, University of Texas Health Science Center at San Antonio).

ß-Galactosidase Assays

B. subtilis strains carrying either pSR5 or an integrated pSGMU31Δ2 plasmid were grown in DS medium and harvested at various times during growth and sporulation. Cells were disrupted by passage through a French pressure Cell (twice at 20,000 lb/in^2) and analyzed for B-galactosidase activity as described by Miller (Miller, 1972).

DNA Sequencing

DNA sequencing was performed by the method of Sanger *et al.* (1977) with the Sequenase reagents (U.S. Biochemical Corp.) and the protocol provided by the manufacturer.

Construction of the *sig*EΔ84 allele.

The *sig*E gene was reconfigured to encode a σ^E-like protein as its direct product. A single-stranded oligonucleotide was hybridized to a region within a single-stranded copy of this fragment (cloned in M13mp19). This resulted in the looping out of the DNA that encodes the precursor element of P^{31}. A complement to the M13-containing DNA was synthesized by using the

oligonucleotide as a primer. Following second-strand synthesis and destruction of the loop by Si nuclease, the DNA mixture was introduced into *E. coli* by transformation. Cloned DNAs which both lost the *HindII* site that is unique to the precursor portion of the sequence and contained a *PstI* fragment of approximately 1.020 bp (rather than the parental 1.100 bp) were picked as putative *sig*E mutants (*sig*EΔ84), expected to contain a sequence in which the region encoding the σ^E amino terminus is positioned immediately downstream of the P^{31} initiation codon and ribosome-binding site. This was verified by sequencing the splice regions of constructions that were positive by the restriction endonuclease criteria.

The splice region of one of the clones diverged from the parental sequence only after the initiating ATG codon. At that point, the sequence encoding the σ^E amino terminus began in frame with the ATG codon.

The 1.02-kbp *PstI* fragment carrying the *sig*EΔ84 allele was cloned into unique *PstI* sites of the plasmid vectors pJM102 and pSI-1 to form pJMΔ84 and pSIΔ84, respectively. The *PstI* site of each of these plasmids lay downstream of an IPTG (isopropylthiogalactopyranoside)-inducible promoter) *Plac* in pJM102 and *Pspac* in pSI-1). Both plasmids replicated in *E. coli,* but only pSI-1 replicated in *B. subtilis*. The *sig*EΔ84 gene was cloned in the proper orientation for expression from the inducible promoters as verified by restriction endonuclease analysis of the ability of this gene to specify a product in *E. coli*.

EXAMPLE I

Production of Sigma Protein in *Escherichia coli*

Cultures of *E. coli* which carried either the mutant or wild-type *sig*E allele under *Plac* or *Pspac* control were induced with IPTG. The amount of P^{31} or $\sigma^E\Delta84$ present at 2 h after induction was determined by Western immunoblot analysis. An autoradiogram of the results of this experiment is presented in Fig. 3. Significant amounts of protein that reacted with the anti-P^{31}/σ^E antibody were present in all of the extracts. The extracts from strains carrying the *sig*EΔ84 allele synthesized a protein ($\sigma^E\Delta84$) with the apparent mobility of σ^E, while the wild-type allele specified a protein with the mobility of P^{31}. In addition to the $\sigma^E\Delta84$ band, extracts prepared from the *sig*EΔ84 strains contained one or more prominent lower-molecular-weight proteins that reacted with the antibody. The lower-molecular-weight proteins varied in abundance in different extract preparations and probably represent breakdown products of σ^E. They were similar in size to but of greater intensity than bands when analyzing extracts of *B. subtilis* cells that synthesize P^{31} and σ^E (Jonas *et al.*, 1989). Densitometry measurements of radioactively labeled antibody bound to the Western blots revealed that the amount of the antibody fixed to the two major bands seen in the *sig*EΔ84 allele encodes a protein with the apparent molecular weight of σ^E but the *sig*EΔ84 product accumulated to a level only 10 to 20% of that obtained from the wild-type *sig*E allele in *E. coli*.

EXAMPLE 2

Effect of the *sig*EΔ84 allele on *B. subtilis*

JH642 was transformed with pJMΔ84. Cells that had integrated this nonreplicating plasmid into their

chromosomes were selected by chloramphenicol resistance. Recombination between pJMΔ84 and its homologous sequences on the *B. subtilis* chromosome could occur either upstream or downstream of the Δ84 deletion. Recombination upstream of the deletion would place the mutant allele under the control of the *spoIIG* promoter, allowing it to be expressed. A recombination event downstream of the mutation would leave a wild-type allele as the expressed copy. The Δ84 deletion lay approximately 200 bp from the upstream end of the 1.02-kpb fragment. It was anticipated that approximately 20% of the chloramphenicol-resistant transformants should have the mutant allele as the expressed copy under conditions of random recombination.

Of the 497 transformants receiving pJMΔ84, 127 (25%) were Spo$^-$ (i.e., colonies remained white and began to lyse after 24 h at 37°C on DS medium). In a similar experiment in which the transforming DNA was pJM102 with a wild-type *sig*E allele (PJME-1), no Spo$^-$ transformants were observed (0 of 463). Southern blot analysis of four Spo$^-$ and four Spo$^-$ clones which had been transformed with pJMΔ84 revealed the wild-type *sig*E allele as part of the *spoIIG* operon in the Spo$^+$ clones and the *sig*EΔ84 allele as the operon component in the Spo$^-$ clones.

The *sig*E gene of JH642 was replaced with *sig*EΔ84 by transforming JH642 (Phe$^-$) with SMY chromosomal DNA (Phe$^+$) plus plasmid pJMΔ84. Phe$^+$ transformants were screened for Spo$^-$ clones. The chromosomal DNAs were then examined by Southern blot analysis. These DNAs contained a *PstI*-generated fragment of 1.03 kbp rather than the parental fragment of 1.1 kpb, which hybridized to a *sig*E-specific DNA probe. One of these clones (SEΔ84-1) was characterized further.

The activity of the σ^E in clone $\sigma^E\Delta 84$-1 was analyzed by using a reporter gene (*lacZ*) fused to a σ^E-dependent promoter (*spoIID*) (Rong *et al.*, 1986). SEΔ84-1 carrying a *spoIID::lacZ* fusion made virtually no β-galactosidase during growth or sporulation in DS medium. Western blot analysis failed to detect any σ^E at times when σ^E is normally abundant. No sigma-type protein was detected in *B. subtilis* when an attempt was made to visualize its product by immunoprecipitation of a radiolabeled product or induction in *B. subtilis* from an spac promoter. Both of these methods generate readily detectable P^{31} and σ^E from the wild-type *sigE* allele.

EXAMPLE 3

Translational Fusions to *sigE*

A translation fusion between *sigE* alleles and the *E. coli lacZ* gene was constructed.

As described in the Materials and Methods section, a 340-bp fragment containing upstream DNA and the coding sequence for the first 55 amino acids of P^{31} was cloned into a plasmid vector (pSGMU31Δ2) so as to create an in-frame fusion between P^{31} and the *E. coli lacZ* gene. A similar fusion was created by using the analogous DNA fragment (256 bp) from *sigE*Δ84. The coding sequence for the first 26 amino acids from the *sigE*Δ84 gene was fused to *lacZ*. The vector used was incapable of replication in *B. subtilis* but carried an antibiotic resistance gene (cat) expressible in *B. subtilis*. Thus, transformation of *B. subtilis* with the fusion plasmids followed by selection for chloramphenicol resistance yielded clones in which the plasmid was integrated into its chromosomal homolog. Integration of the plasmid resulted in a Spo$^-$ phenotype owing to the positioning of the *sigE::lacZ*

fusion gene within the spoIIG operon in place of the wild-type allele.

When the wild-type *sigE::lacZ* fusion plasmid was introduced into a *sigE*$^+$ *B. subtilis* strain, all of the clones (38 of 38) displayed a Lac$^+$ phenotype on plates (Table 1). In a similar experiment, only 25% (8 of 32) of the transformants receiving the *sigE*Δ84*::lacZ* DNA were Lac$^+$. As was the case in the previous transformation experiment, the integration event of *::lacZ* into *sigE* could take place either upstream or downstream of the Δ84 mutation. Recombination downstream of the mutation would result in creation of a wild-type *sigE::lacZ* gene. It was therefore possible that the Lac$^+$ clones represented *sigE*$^+$*::lacZ* fusions and that *sigE*Δ84*::lacZ* clones were Lac$^-$. A Southern blot analysis of the chromosomes of a representative sample of Lac$^+$ and Lac$^-$ transformants that had received *sigE*Δ84*::lacZ* DNA was performed. Six of six Lac$^+$ clones had a DNA fragment of the size anticipated for wild-type *sigE* upstream of the *lacZ* gene, and six of six of the Lac$^-$ clones had a fragment of the size expected for *sigE*Δ84 upstream of *lacZ*.

To verify that a Lac$^-$ phenotype is a consequence of the *sigE*Δ84 sequence being upstream of the *lacZ* gene the transformation into SE84-1, a strain which already carries the *sigE*Δ84 allele was repeated. In these experiments, where the creation of a wild-type *sigE*Δ84*::lacZ* fusion was impossible, all of the chloramphenicol-resistant transformants that received the *sigE*Δ84*::lacZ* DNA were Lac$^-$ (25 or 25), while approximately 15% (4 of 27) of the transformants which received the *sigE*Δ84*::lacZ* DNA were Lac$^-$. Analysis of the level of B-galactosidase in extracts prepared from *sigE*Δ84*::lacZ* transformants that were phenotypically Lac$^-$ on plates failed to reveal measurable levels of B-galactosidase. Thus, synthesis of the fusion protein

mirrors the inability of *sig*EΔ84 to form σ^E itself. In both cases, no product was detectable by the assay systems employed.

TABLE 1

Transformation of *B. subtilis* by *sig*E::*lac*Z DNAs*

Recipient	Donor DNA fusion	No. of colonies				
		Total Examined	Spo$^+$	Spo$^-$	Lac$^+$	Lac$^-$
JH642	*sig*E::*lac*Z	38	38	0	38	0
	*sig*EΔ84::*lac*Z	32	32	0	8	24
SE84	*sig*E::*lac*Z	27	27	0	23	4
	*sig*EΔ84::*lac*Z	25	25	0	0	25

[a] Competent recipient cells were transformed with plasmid DNA. Transformants were selected on LB plates supplemented with chloramphenicol (5 μgml). Cm^r clones were replica plated onto DS plates (22) with and without an agar overlay containing X-gal (5-bromo-4-chloro-3-indolyl-ß-/d-thiogalactopyranoside)(0.5 mg/ml)(18). Spo$^+$ colonies turned brown after 24 hr at 37°C on DS plates, while Spo$^-$ colonies remained white. Lac$^+$ colonies turned blue on DS with X-gal by 18 hr at 37°C.

The cloned *sig*E gene was mutagenized by using an oligonucleotide (30-mer) that would generate an altered *sig*EΔ84 with a Lys codon (AAA) between the initiation codon (AUG) and the codon specifying the σ^E amino terminus (GGC). Mutant clones were isolated, and their structures were verified by DNA sequencing.

When introduced into *B. subtilis*, *sig*EΔ81 behaved identically to *sig*EΔ84. It conferred a Spo$^-$ phenotype on cells which carried it (i.e., when *sig*EΔ84 was cloned

into pJM102 and transformed into JH642, approximately 20% of the chloramphenicol-resistant transformants were Spo$^-$), and cells into which the *sig*EΔ81 allele had been congressed synthesized no detectable σ^EΔ81 protein. Thus, the addition of AAA at position two of *sig*EΔ81 yielded no measurable increase in σ^E levels.

During the course of the mutagenesis protocol a *sig*E mutant was generated (*sig*EΔ48) that encoded a protein with the four amino acids of the amino terminus of *sig*EΔ81 joined by an Arg residue to residue 22 of P^{31}. The *sig*EΔ48 mutation was initially identified by the intermediate size of the *PstI* DNA fragment which carried it. Its structure was determined by DNA sequencing.

When *sig*EΔ48 was moved into *B. subtilis*, the cells became Spo$^-$. However, unlike *sig*EΔ84 and *sig*EΔ81, *sig*EΔ48 synthesized an active σ^E-like protein. The *sig*EΔ48 product was seen in Western blots, and its activity was evident by the activation of the spoIID promoter. The activation of the spoIID promoter by *sig*EΔ48 does not apparently require the processing of σ^EΔ48 into the wild-type σ^E protein. Transcription of spoIID occurred in *B. subtilis* strains carrying *sig*EΔ48 even if the strain had a second mutation in a gene (spoIIGA) that is essential for processing. In addition, no change was detected in the mobility of σ^EΔ48 at times in sporulation (t_4) when the processing activity should be present. Thus, σ^EΔ48 is both insensitive to processing and active without processing. The level of product present in the *sig*EΔ48 strain was comparable to that found in the wild-type *sig*E strain.

Extracts were produced from *B. subtilis* strains lacking a chromosomal copy of *sig*E but containing plasmid-borne copies of either the wild-type (*sig*E$^+$) or one of the mutant alleles of *sig*E (pSR51 or pSR54). Western blot

analysis for proteins of the size of either P^{31} or σ^E-sized proteins in this system indicated no detectable proteins in the extracts prepared from the strains with the mutant *sig*E alleles. This result was the same obtained with the *sig*EΔ84 and *sig*EΔ81 alleles.

EXAMPLE 4

β-galactosidase Fusion Proteins

B. subtilis strains expressing β-galactosidase fusion proteins were constructed as described (Jonas *et al.*, 1990). The *E. coli lac*Z gene was translationally coupled to *sig*E by cloning a 340-bp *PstI-SauIIIA1* fragment of the 1.1 kbp cloned *sig*E gene into *PstI-BamHI*-cut pSGMU31Δ2. This coupled the 5′ end of *lac*Z in frame to *sig*E at a site 165 bp downstream of the sequence encoding the amino terminus of P^{31}. In a similar fashion, the bp segment encoding the polypeptide having the amino acid sequence of Figure 1 was cloned into this vector. This construct was formed at the same site within *sig*E but had only the base pairs shown in Figure 2 of the *sig*E protein-coding region upstream of *lac*Z. JH642 was transformed with pSGMU31Δ2 containing the coding sequence for either the *sig*E^+ or *sig*EΔ84-encoded amino terminus. Clones in which the plasmid had integrated into the *B. subtilis* chromosome were selected by chloroamphenicol resistance (2.5 μg/ml) and screened by Southern blot analysis for integration into the *sig*E locus with the proper *sig*E allele positioned upstream of *lac*Z.

The half-life of the fusion proteins expressed from transformed *Escherichia coli* or *B. subtilis* host cells were measured. Table 2 shows the results.

TABLE 2

Protein	Microorganism	Half Life
Sigma E	*E. coli*	ND
β-galactosidase	*E. coli*	30 min
β-galactosidase	*B. subtilis*	ND
Sigma E:SS'	*E. coli*	>2 hr
β-galactosidase:SS[1]	*E. coli*	
β-galactosidase:SS[1]	*B. subtilis*	>2 hr

[1] SS is the 29AA sequence shown in Figure 1.

EXAMPLE 5

Structural Perturbations in Protein Stabilization Sequence

The effect of perturbation of α-helical structure of the protein stabilization sequence shown in Figure 1 was examined by altering the primary amino acid sequence.

The DNA sequence shown in Figure 2 was altered by insertion of an arginine codon at position 10, resulting in a new sequence encoding a 30-amino acid polypeptide. In order to effect the insertion, a restriction site was created at codon position 11 by substituting codon CTA for CTC. The base pair segment coding for the 30-amino acid polypeptide and a gene segment encoding β-galactosidase were cloned. Expression of β-galactosidase from a *B. subtilis* bacterial host cell was examined by Western blot. Amounts of expressed fusion polypeptide were less than measured in systems utilizing the 29-amino acid stabilization sequence.

The hydrophobic and hydrophilic faces of the α-helix formed by the 30-amino acid polypeptide were twisted relative to each other by about 100°.

Additional insertions into the DNA sequence encoding the 30 amino acid polypeptide were made. Codons CTC and GCC encoding leucine and alaline were inserted between codon 10 and codon 11 of the base sequence, resulting in a 180° twist of the polypeptide α-helix structure as compared with the α-helix of the 29 amino acid polypeptide structure. The new 32 amino acid polypeptide was expressed as a fusion polypeptide with β-galactosidase from a transformed *B. subtilis* host. Expression was less efficient than with fusions formed from either the 29 or the 30 amino acid protein stabilizing sequence, as indicated by Western blot analysis.

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CLAIMS

1. A protein stabilization sequence comprising an amino acid sequence attached to a protein to be stabilized, said sequence including a number of amino acid residues sufficient to form a structure having an outwardly directed hydrophobic face and a positively charged polar face.

2. The protein stabilization sequence of claim 1 wherein the structure is an α-helix.

3. The protein stabilization sequence of claim 1 having up to 100 amino acid residues.

4. The protein stabilization sequence of claim 1 wherein the outwardly directed hydrophobic face is in alignment with the positively charged face.

5. The protein stabilization sequence of claim 1 wherein the amino acid sequence is from about 10 to about 50 amino acid residues.

6. The protein stabilization sequence of claim 1 wherein the amino acid sequence is 29 amino acid residues.

7. The protein stabilization sequence of claim 6 wherein the sequence comprises the sequence of Figure 1 or a biologically functional equivalent thereof.

8. A method of stabilizing proteins comprising attaching the protein stabilization sequence of claim 1 to a proteolytically sensitive protein.

9. The method of claim 8 wherein the attaching is to an amino terminal end of a proteolytically sensitive protein.

10. The method of claim 8 wherein the attaching is to a carboxy terminal end of a proteolytically sensitive protein.

11. The method of claim 8 wherein the attaching is to a proteolytically sensitive protein at both terminii.

12. The method of claim 8 wherein the proteolytically sensitive protein is RNA polymerase sigma factor.

13. The method of claim 12 wherein the RNA polymerase sigma factor is from *B. subtilis* or *E. coli*.

14. A method of enhancing stability of recombinant proteins comprising

obtaining a gene segment encoding the protein stabilization sequence of claim 1;

preparing a recombinant vector having a gene encoding the protein stabilization sequence and a gene encoding a protein to be stabilized;

transforming a host cell with the recombinant vector;

selecting transformed host cell colonies; and

facilitating expression of a heterologous protein fused N- terminally with the protein stabilization sequence.

15. The method of claim 14 wherein the protein to be stabilized is β-galactosidase.

16. The method of claim 14 wherein the host cell is a bacterium.

17. The method of claim 14 wherein the host cell is *B. subtilis* or *E. coli*.

18. A system for production of a stable, heterologous protein in prokaryotic hosts comprising DNA having a coding sequence for the protein stabilization sequence of claim 1 operably linked to and out of reading frame with a terminal leader DNA sequence encoding a protein expressible as an active fusion protein.

19. A DNA segment which codes for the protein stabilization sequence of claim 1 or a functionally stabilizing equivalent thereof.

20. The DNA segment of claim 19 having the base pair sequence of Figure 2 or a functionally stabilizing structural equivalent thereof.

21. A recombinant DNA vector comprising a first base sequence coding for the protein stabilization sequence of claim 1 or a second base sequence complementary to the first base sequence.

```
                                                                  Pro
                                                                   ⇩
Met Lys Lys Leu Lys Leu Arg Leu Thr His Leu Trp
 1                                  10  11
                Pro
                 ⇩
Tyr Lys Leu Leu Met Lys Leu Leu Gly Leu Lys Ser Asp
                15                  20
Glu Val Tyr Tyr Ile
                29
```

FIG. 1

```
ATG AAA AAA CTG AAA TTA CGG TTG ACG CAC CTC TGG
 1                              10
TAT AAG CTG CTG ATG AAA CTT GGG CTG AAA AGT GAT
                               20
GAA GTC TAT TAC ATA
```

FIG. 2

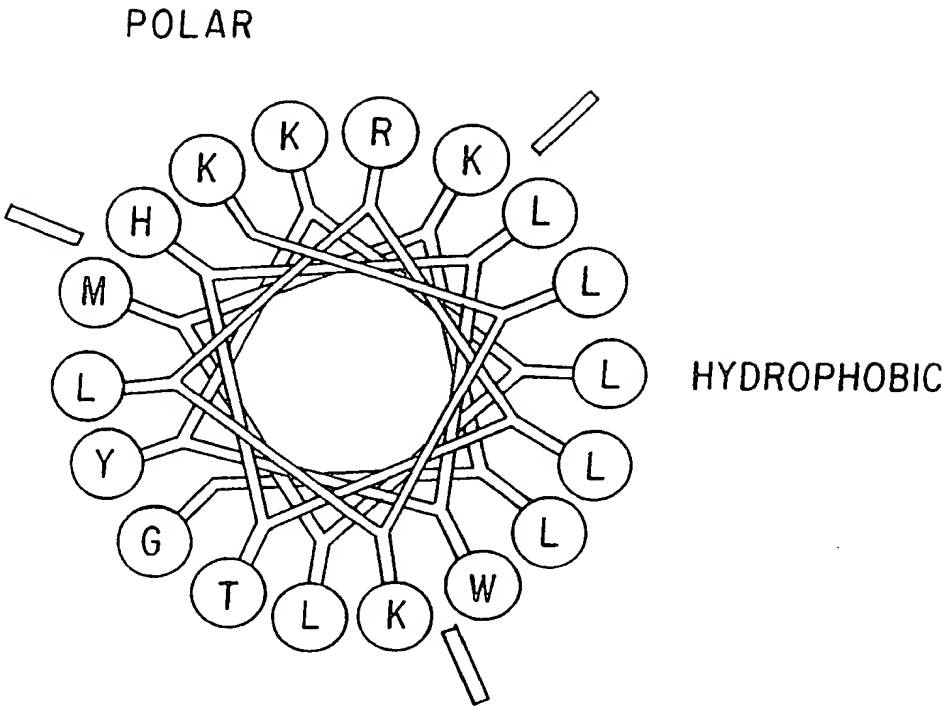

FIG. 3

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 92/05810

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/62; C12N9/38; C12N15/75; C07K7/10

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N ; C07K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	J. BACTERIOLOGY vol. 172, no. 8, August 1990, AM. SOC. MICROBIOL., BALTIMORE, US; pages 4178 - 4186 R.M. JONAS ET AL. 'Phenotypes of Bacillus subtilis mutants altered in the precursor-specific region of sigma E'	1-7, 19-21
Y	see page 4179, right column, line 14 - line 30 see page 4181, right column, line 27 - line 38 see page 4185, right column, line 41 - line 47	8-18

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° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
06 OCTOBER 1992	26. 10 92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	HORNIG H.

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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

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Y	BIO/TECHNOLOGY vol. 7, no. 2, February 1989, NATURE AMERICA, INC., NEW YORK, US H.HEELEBUST ET AL. 'Different approaches to stabilize a recombinant fusion protein' see page 167, left column, line 6 - page 168, left column, line 3; figures 1-5 ---	1,8-18
Y	EP,A,0 098 118 (BERMAN,SILHAVY,WEINSTOCK) 11 January 1984 see page 4, line 34 - page 7, line 5; claims 1-18 ---	1,18
P,X	J. BACTERIOLOGY vol. 173, no. 24, December 1991, AM. SOC. MICROBIOL., BALTIMORE, US; pages 7821 - 7827 H.K.PETERS III AND W.G. HALDENWANG 'Synthesis and fractionation properties of SpoIIGA, a protein essentiell for pro-sigma E processing in Bacillus subtilis' see page 7824, left column, line 38 - page 7826, left column, line 4; figure 1 ---	1,2, 19-21
P,X	J. BACTERIOLOGY vol. 174, no. 14, July 1992, AM. SOC. MICROBIOL., BALTIMORE, US; pages 4629 - 4637 H.K.PETERS III ET AL. 'Mutational analysis of the precursor-specific region of Bacillus subtilis sigma E' see page 4630, right column, line 61 - page 4635, right column, line 15; figures 1,5 -----	1-18

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. US 9205810 SA 62391

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0098118	11-01-84	US-A- 4503142	05-03-85

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